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Original Article

Select small nucleolar RNAs in blood components as novel biomarkers for improved identification of comorbid traumatic brain injury and post-traumatic stress disorder in veterans of the conflicts in Afghanistan and Iraq

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Abstract: Background: The present study was designed to validate the ability of our recently identified set of small noncoding RNA candidate mild traumatic brain injury (mTBI) biomarkers to diagnose mTBI in the presence or absence of post-traumatic stress disorder (PTSD) comorbidity. Using qPCR, we explored the regulation of the candidate biomarkers in peripheral blood mononuclear cells (PBMC) from 58 veterans. Results: We confirmed that 4 small nucleolar RNAs (snoRNAs), ACA48, U35, U55, and U83A, are significantly down-regulated in PBMC from veterans with mTBI and PTSD compared to non-TBI, control subjects with PTSD only. We found that the snoRNA biomarkers are able to dissect subjects with comorbid mTBI and PTSD from PTSD subjects without mTBI with 100% sensitivity, 81% accuracy, and 72% specificity. No significant differential expression of snoRNA biomarkers was found in mTBI subjects without comorbid PTSD. However, we found significantly lower U55 contents in subjects with PTSD. We explored the regulation of ACA48 in rodent models of PTSD or blast-induced mTBI to gather proof-of-concept evidence that would connect the regulation of the biomarkers and the development of mTBI or PTSD. We found no change in the regulation of ACA48 in the mTBI rat model. We did, however, find significant down-regulation of ACA48 in the PTSD mouse model 24 hours following psychological trauma exposure. This may reflect a short-term response to trauma exposure, since we found no change in the regulation of ACA48 in veteran PTSD subjects 3.6 years post-deployment. Conclusions: Additional application of the 4 snoRNA biomarker to current diagnostic criteria may provide an objective biomarker pattern to help identify veterans with comorbid mTBI and PTSD. Our observations suggest that biological interactions between TBI and PTSD may contribute to the clinical features of veterans with comorbid mTBI and PTSD. Future investigations on mTBI mechanisms or TBI biomarkers should consider their interactions with PTSD.

Keywords: Mild traumatic brain injury, post-traumatic stress disorder, biomarker, small nucleolar RNAs

Introduction

Traumatic brain injury (TBI) has been referred to as the signature injury of veterans of the wars in Iraq and Afghanistan [1]. The majority of documented TBI cases among service members returning from Operation Enduring Freedom and Operation Iraqi Freedom (OEF/OIF) are characterized as mild-TBI (mTBI) [2]. Neurological and neuropsychiatric complaints,

including mood changes and deficits in memory or attention, are very common in mTBI subjects. Current evidence suggests that oxidative stress and inflammatory responses following TBI play key roles in the accumulated cellular damage seen in TBI subjects [3]. Axonal damage due to mechanical shearing forces in the brain during the injury also contributes to the disruption in neuronal function and connectivity in the brains of TBI patients [4]. Accumulation of neuropathologic features associated with abnormal processing of the microtubule-associated protein, tau, may also contribute to long-term TBI complications [5].

TBI is associated with an increased risk for developing post-traumatic stress disorder (PTSD) [6, 7], an anxiety disorder that may result from exposure to trauma. This may be due, in part, to an inability to suppress attention to trauma-related stimuli as a result of TBI-mediated neuronal damages [8]. Chronic inflammation in individuals that sustained a TBI may also contribute to PTSD [9, 10]. The significant overlap of the symptoms associated with mTBI and comorbid PTSD complicates accurate mTBI evaluation and prognosis [6]. The current lack of easily accessible and accurate diagnostic biological fingerprints able to monitor mTBI clinical stages often impedes correct classification of mTBI/PTSD disease state, and thus the timely use of appropriate intervention. This has serious implications for veterans, since an estimated 7% (~119, 720) of troops returning from Iraq and Afghanistan suffer from both TBI and PTSD [11]. Veterans with history of TBI and comorbid PTSD complain of more severe clinical complications, including neuropsychiatric symptoms (e.g., anxiety and depression) and neurocognitive dysfunctions, and therefore require more immediate intervention and support.

We previously reported that a set of thirteen small noncoding RNA candidate mTBI biomarkers have significantly lower levels of expression in accessible peripheral blood mononuclear cells (PBMC) from veterans with a history of mTBI compared to non-TBI control veterans [12]. However, the majority of mTBI and control subjects in our initial study had comorbid PTSD. Based on this consideration, the present studies were designed to validate, in a new veteran cohort, the ability of these candidate biomarkers to distinguish mTBI in the presence or absence of PTSD comorbidity.

Materials and methods

Study cohort

58 OIF and OEF veteran cases (6 mTBI/PTSD, 11 non-TBI/PTSD, 7 mTBI/non-PTSD, and 34 non-TBI/non-PTSD) were recruited by The War Related Illness and Injury Study Center (WRIISC), Department of Veterans Affairs, New Jersey Health Care System (DVANJHCS), East Orange, NJ. Male and female participants were included if they were between 18-75 years of age and had completed a clinical evaluation at the New Jersey WRIISC. Participants were included regardless of their mTBI history. Cases with intercurrent infections or inflammatory-related conditions were excluded. Participants were classified as having a history of mTBI if they met at least one of 4 criteria on the veteran traumatic brain injury screening tool (VAT-BIST) [13], and had a score at least one standard deviation below the norm for age and education on the Repeatable Battery for Neuropsychological Testing (RBANS) [14]. Classification criteria for control cases included a negative VAT-BIST score and a RBANS score less than one standard deviation below the norm. Human participants were recruited by the WRIISC following voluntary written informed consent. The study was approved by the DVANJHCS Internal Review Board.

PBMC isolation

Blood specimens from human subjects were collected by venipuncture and drawn into BD Vacutainer CPT Cell Preparation Tubes (Becton, Dickinson and Company). PBMCs were isolated from freshly collected blood specimens according to the manufacturer's instructions, and were stored at -80°C until use.

RNA preparation

Total RNA was isolated from approximately 10-50 mg of PBMCs using RNA STAT-60, according to the manufacturer's instructions (Tel-Test, Friendswood, TX, USA).

Quantitative real-time polymerase chain reaction (qPCR) studies

Contents of targeted candidate small noncoding RNA biomarkers in PBMC specimens were assessed by qPCR using custom-designed

qPCR primer sets, as we have previously done [12]. Data were normalized relative to those for the 5.8S ribosomal RNA. Levels of targeted small non-coding RNA were expressed relative to those in control groups using the $2^{-\Delta\Delta Ct}$ method [15].

Biomarker assessments in experimental rodent models of mTBI or PTSD

Blast-induced rat model of mTBI: Adult male Long Evans hooded rats (250-350 g; 10-12 weeks of age) were exposed to overpressure injury using the Walter Reed Army Institute of Research shock tube, which simulates the effects of air blast exposure under experimental conditions [16]. Rat blood specimens were collected 1.3 years following blast exposure via saphenous vein puncture. Parallel studies using age, gender, and strain-match rats without air blast exposure served as controls. All animal studies were approved by the James J. Peters Department of Veterans Affairs Medical Center (Bronx, NY), The Walter Reed Army Institute of Research and Naval Medical Research Center Institutional Animal Care and Use Committee, and the Icahn School of Medicine at Mount Sinai, (NY, New York) IACUC and IRB.

Chronic social defeat mouse model of PTSD: C57BL/6J mice were exposed to social defeat (SD) sessions once a day for 10 consecutive days, as previously described [17]. Twenty-four hours after the last (day 10) social defeat session, post-chronic social defeat blood specimens were collected by sub-mandibular bleeding. Parallel studies using age, gender, and strain-match animals not exposed to the social defeat protocol served as controls.

Assessments of small noncoding RNA biomarkers: Total RNA, including small RNA, were extracted from the blood using the PAXgene Blood miRNA Kit (PreAnalytiX, Qiagen), following the manufacturer's instructions. qPCR primers for mouse ACA48 and 5.8S rRNA were custom-designed and synthesized by Applied Biosystems. 10 ng of RNA was used in each reaction in order to prepare cDNA, using the Taqman MicroRNA Reverse Transcription Kit. Data were normalized to those for 5.8S rRNA using the $2^{-\Delta\Delta Ct}$ method. Levels of target small non-coding RNA were expressed relative to those in the control groups.

Results

Recruitment of 58 OEF/OIF cases for the biomarker study

A total of 58 OIF/OEF veterans were recruited to test the value of our previously identified panel of 13 candidate biomarkers, either individually or in combination, to distinguish mTBI in the presence or absence of PTSD comorbidity. This study cohort is comprised of 13 mTBI cases and 45 non-TBI cases. The proportion of veterans (22%) classified with mTBI in our recruited cohort is consistent with, and even slightly above, previous prevalence estimates of 12% reported in a cross-sectional survey of 2,235 active duty, guard, and reserve OEF/OIF veterans [18]. Demographic information for the study cohort is shown in **Table 1**. There were no significant differences between the mTBI and non-TBI groups with respect to group average values for age (31.2 ± 8.6 and 34.8 ± 10.3 years, respectively; p=0.25) or years of education (14.2 ± 2.3 and 14.1 ± 2.1 years, respectively; p=0.83). The mTBI group had a significantly longer time interval between their last deployment and their recruitment into this study (4.6 ± 2.3 for the mTBI group and 2.8 ± 2.3 years for the non-TBI group, p=0.02). The proportion of males in the mTBI and the non-mTBI groups were 92% and 87%, respectively. Notably, our study cohort contained mTBI and non-TBI cases with or without co-morbid PTSD. This presents the opportunity to explore the ability of our previously identified candidate biomarkers to distinguish mTBI in the presence or absence of PTSD comorbidity.

PBMC expression of the candidate biomarkers in mTBI and control non-TBI cases comorbid with PTSD

We previously identified 13 candidate small noncoding RNA mTBI biomarkers in an exploratory biomarker discovery study cohort of mTBI and non-TBI cases that were predominately comorbid with PTSD [12]. These 13 candidate small noncoding RNA mTBI biomarkers are listed in **Figure 1A**. In this study, we continued to test for potential differentiation of these 13 small noncoding RNA biomarkers among mTBI cases with PTSD comorbidity, compared to control non-TBI cases with PTSD (herein referred to as mTBI/PTSD and non-TBI/PTSD cases). Our current study cohort contained 6 mTBI/PTSD

Table 1. Demographic characteristics of the study cohort

Case No.	mTBI/non-TBI	Age	Sex	Ethnicity	Interval (yrs) since last deployment	Education (yrs)	Comorbidity
33361	mTBI	26	F	Wh/Hisp	3.2	13	Yes
34084	mTBI	32	M	Bl/Afr Am/nHisp	3.5	13	Yes
34119	mTBI	29	M	Wh/nHisp	8.1	18	Yes
34124	mTBI	47	M	Wh/nHisp	2.6	18	Yes
34193	mTBI	51	M	Wh/nHisp	6.7	13	Yes
34260	mTBI	28	M	Bl/Afr Am/nHisp	7.9	13	Yes
33478	mTBI	30	M	Wh/nHisp	6.4	18	No
33570	mTBI	23	M	Wh/Hisp	2.8	13	No
34055	mTBI	28	M	Wh/Hisp	3.2	14	No
34128	mTBI	24	M	Wh/nHisp	1.5	12	No
34138	mTBI	26	M	Wh/nHisp	2.6	12	No
34254	mTBI	35	M	Wh/nHisp	7.2	15	No
34261	mTBI	26	M	Wh/nHisp	3.7	13	No
33759	non-TBI	28	M	Wh/Hisp	0.4	12	Yes
33899	non-TBI	60	M	Wh/nHisp	not deployed	14	Yes
33913	non-TBI	22	F	Bl/Afr Am/nHisp	1.0	12	Yes
33944	non-TBI	30	M	Wh/nHisp	8.4	14	Yes
33848	non-TBI	25	M	Bl/Afr Am/nHisp	4.3	13	Yes
33977	non-TBI	42	M	Bl/Afr Am/nHisp	2.3	14	Yes
34060	non-TBI	27	F	Wh/nHisp	3.9	17	Yes
34070	non-TBI	24	M	Wh/Hisp	1.2	12	Yes
34079	non-TBI	37	M	Wh/nHisp	4.3	14	Yes
34087	non-TBI	40	M	Wh/Hisp	4.4	12	Yes
34160	non-TBI	29	M	Wh/nHisp	6.0	12	Yes
30597	non-TBI	40	M	Wh/nHisp	4.4	18	No
31429	non-TBI	39	M	Bl/Afr Am/nHisp	0.9	14	No
31824	non-TBI	43	M	Bl/Afr Am/nHisp	6.5	13	No
33673	non-TBI	25	M	Wh/Hisp	2.8	14	No
33676	non-TBI	32	M	Wh/Hisp	1.0	16	No
33699	non-TBI	42	M	Bl/Afr Am/nHisp	0.7	16	No
33765	non-TBI	26	F	Wh/Hisp	0.9	16	No
33786	non-TBI	52	M	Bl/Afr Am/nHisp	0.7	14	No
33819	non-TBI	24	M	Wh/Hisp	1.9	12	No
33832	non-TBI	43	M	Wh/nHisp	4.4	12	No
33858	non-TBI	29	M	Bl/Afr Am/nHisp	6.5	18	No
33863	non-TBI	41	M	Wh/nHisp	0.8	12	No
33864	non-TBI	23	M	Wh/Hisp	1.8	14	No
33877	non-TBI	59	M	Wh/nHisp	0.7	16	No
33904	non-TBI	22	M	Asian	0.6	12	No
33905	non-TBI	38	M	Wh/nHisp	1.1	18	No
33911	non-TBI	29	M	Bl/Afr Am/nHisp	7.7	16	No
33915	non-TBI	25	F	Bl/Afr Am/nHisp	4.2	14	No
33918	non-TBI	33	M	Wh/Hisp	1.7	12	No
33958	non-TBI	23	M	Wh/Hisp	1.2	12	No
33992	non-TBI	28	M	Wh/nHisp	4.7	13	No
34026	non-TBI	34	M	Asian	1.3	13	No
34047	non-TBI	45	F	Bl/Afr Am/nHisp	4.9	15	No

34076	non-TBI	40	M	Bl/Afr Am/nHisp	0.7	12	No
34077	non-TBI	25	M	Wh/Hisp	1.2	13	No
34093	non-TBI	47	M	Wh/nHisp	1.9	16	No
34094	non-TBI	42	M	Wh/nHisp	3.8	17	No
34117	non-TBI	42	M	Wh/nHisp	0.4	20	No
34120	non-TBI	54	M	Wh/nHisp	5.2	16	No
34126	non-TBI	34	M	Wh/Hisp	6.8	13	No
34142	non-TBI	46	M	Wh/Hisp	1.7	14	No
34192	non-TBI	24	M	Wh/Hisp	1.6	12	No
34247	non-TBI	26	F	Wh/Hisp	1.0	13	No
33856	non-TBI	27	M	Wh/Hisp	0.6	12	No

Information is presented for the 58 cases recruited for this study. Cases are subgrouped by characterization of mTBI with comorbid PTSD, non-TBI with PTSD, non-TBI without PTSD, and non-TBI without PTSD. Abbreviations: Wh, white; Hisp, hispanic; nHisp, non-hispanic; Bl, black; Afr Am, African American.

and 11 non-TBI/ PTSD cases (**Table 1**). There were no significant differences between the mTBI/PTSD and non-TBI/PTSD groups with respect to group average values for age (31.5 ± 10.7 and 33.1 ± 11.08 years, respectively; p=0.67), post-deployment interval (5.3 ± 2.5 and 3.6 ± 2.5 years, respectively; p=0.20), or years of education (14.7 ± 2.5 and 13.3 ± 2.6 years; p=0.18). The proportion of males in the mTBI and the non-mTBI groups were 83% and 82%, respectively.

We quantified PBMC contents of individual candidate small noncoding RNA mTBI biomarkers in each of the cases, using qPCR. Consistent with observations from our initial exploratory biomarker discovery studies [12], we that found that 4 of the small noncoding RNA biomarkers, ACA48, U35A, U55, and U83A, are significantly down-regulated in mTBI/PTSD cases compared to non-TBI/PTSD cases (**Figure 1B**). Our observations validate, for the first time, ACA48, U35A, U55, and U83A in PBMC as biomarkers of mTBI in the context of PTSD comorbidity. Moreover, using unsupervised hierarchical cluster analysis, we found that a combination of ACA48, U35A, U55, and U83A was able to correctly distinguish mTBI/PTSD cases from control non-TBI/PTSD cases with 82% accuracy, 100% sensitivity, and 72% specificity (**Figure 1C**, **1D**). The sensitivity and specificity of using the combined biomarker for correctly segregating mTBI/PTSD vs. non-TBI/PTSD cases in our present study cohort was confirmed using the receiver operating characteristic (ROC) analysis as an independent assessment.

We note that all 4 validated biomarkers of mTBI/PTSD comorbidity are members of the small nucleolar RNA (snoRNA) class that are known for their activities in modulating RNA splicing, stability, and/or translation [19-21]. Potential relevance of the 4 snoRNA biomarkers to TBI/PTSD pathophysiology will be discussed in more details in the *Discussion* section, below.

PBMC expression of ACA48, U35A, U55, and U83A in mTBI and control non-TBI cases without PTSD

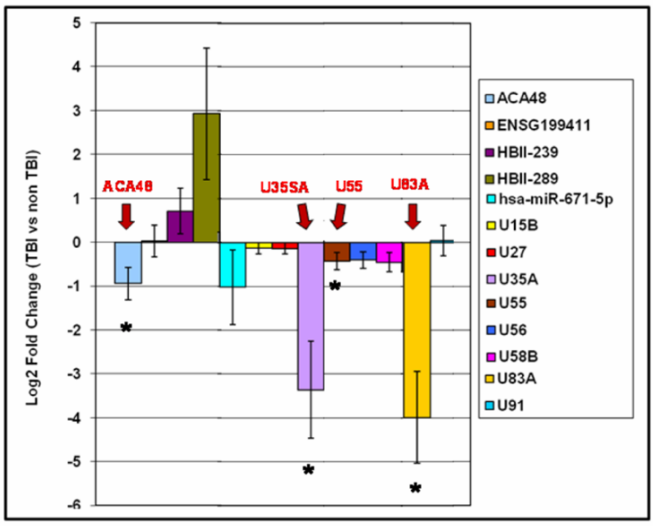
We continued to explore the regulation of the 4 snoRNA biomarkers in mTBI and non-TBI cases in the absence of PTSD. Our study cohort of 58 veteran cases contained 7 mTBI cases without PTSD and 34 non-TBI cases without PTSD (herein referred to as mTBI/non-PTSD and non-TBI/non-PTSD cases, respectively). Demographic information for the mTBI/non-PTSD and non-TBI/non-PTSD cases is shown in **Table 1**. As a group, mTBI/non-PTSD cases were significantly younger than control non-TBI/non-PTSD cases (group averages 27.4 ± 4.1 and 35.3 ± 10.05 years, respectively; *p*-value 0.05). There were no significant differences between the mTBI/non-PTSD and non-TBI/non-PTSD groups with respect to group average values for post-deployment interval (3.9 ± 2.1 and 2.5 ± 2.2 years, respectively; p=0.15) or years of education (13.86 ± 2.1 and 14.4 ± 2.2 years, respectively; p=0.59). The proportion of males in the mTBI and the non-TBI groups were 100% and 88%, respectively.

We found no significant differences in PBMC contents of ACA48, U35A, U55, or U83A in mTBI vs. non-TBI cases in the absence of PTSD comorbidity (**Figure 2A**). Moreover, we found that these 4 snoRNA biomarkers have no predictive value for segregating mTBI and non-TBI

A

Candidate small RNA Biomarker species	mTBI/PTSD vs. non-TBI/PTSD
ENSG00000199411	0.47
hsa-miR-671-5p	0.50
ACA48	0.05
HBII-239	0.35
HBII-289	0.19
U15B	0.63
U27	0.72
U35A	0.05
U55	0.05
U56	0.14
U58B	0.12
U83A	0.007
U91	0.91

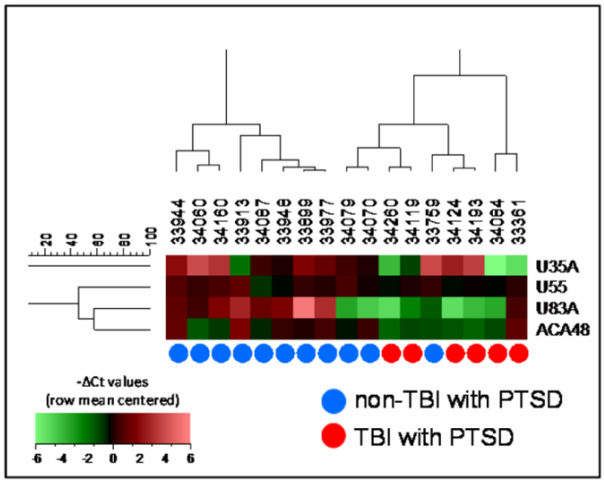
B



C

Biomarker combinations	TP	FP	TN	FN	sensitivity	95% CI	specificity	95% CI	accuracy
ACA48/U35A/U55/U83A	6	3	8	0	1.00	0.51-1.00	0.72	0.39-0.93	0.82
ACA48/U35A/U83A	6	2	9	0	1.00	0.51-1.00	0.81	0.47-0.97	0.88
U35A/U55/U83A	6	2	9	0	1.00	0.51-1.00	0.81	0.47-0.97	0.88
ACA48/U55/U83A	5	3	8	1	0.83	0.36-0.99	0.72	0.39-0.93	0.76
ACA48/U35A/U55	4	3	8	2	0.66	0.24-0.94	0.72	0.39-0.93	0.71
U35A/U83A	6	2	9	0	1.00	0.51-1.00	0.81	0.47-0.97	0.88

D



E

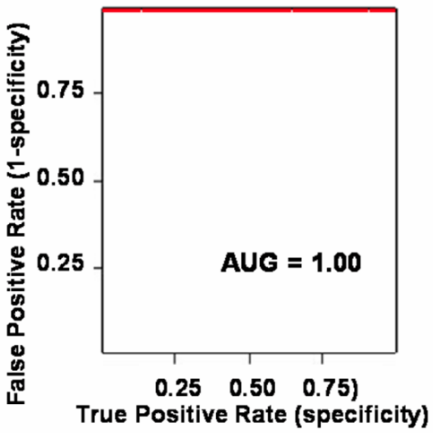
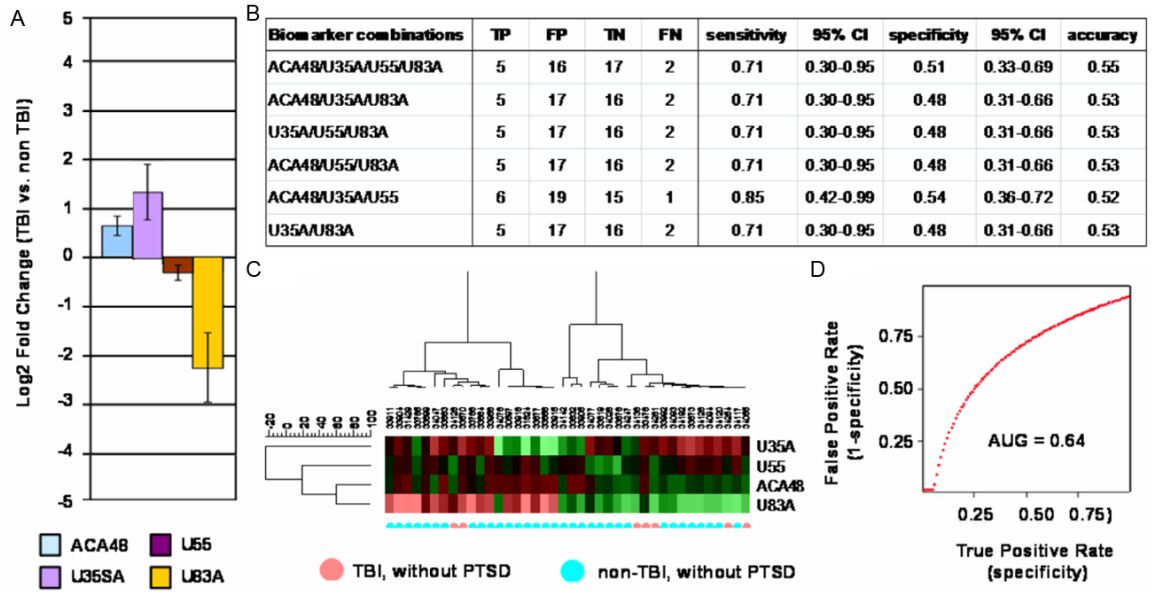


Figure 1. Candidate mTBI small noncoding RNA biomarker expression in TBI vs non-TBI cases in the context of PTSD comorbidity. A, B. Contents of candidate small noncoding RNA biomarkers in PBMC from mTBI/non-PTSD and non-TBI/non-PTSD cases were assessed by qPCR. A. t-test *p*-value comparisons between mTBI/PTSD vs. non-TBI/PTSD cases. Red letters highlight candidate small noncoding RNA mTBI biomarkers with significant differential regulation in PBMC specimens of mTBI/PTSD compared to non-TBI/PTSD cases. B. Bar graphs show mean ± SEM fold change (in Log2 values) of candidate mTBI biomarkers in mTBI/PTSD vs. control non-TBI/PTSD cases. Values <0 or >0 indicate, respectively, down-regulation or up-regulation in mTBI/PTSD compared to non-TBI/PTSD cases. Arrows point to the 4 small noncoding RNAs that were significantly down-regulated (*p<0.05) in mTBI/PTSD vs. control non-TBI/PTSD cases. C-E. The combination of a 4-biomarker panel, ACA48, U35A, U55, and U83A, provides a sensitive and specific criterion for differentiating mTBI/PTSD from non-TBI/PTSD cases. Unsupervised hierarchical clustering of mTBI/PTSD and non-TBI/PTSD cases was conducted using the Unweighted Pair Group Method with Arithmetic Mean agglomerative method. Cluster assignment was based on assigning samples into one of two major clusters. One of the major clusters contained a majority of the mTBI samples and was designated as the mTBI clus-

ter, while the other major cluster was designated as the non-TBI cluster. A. Summation table of the unsupervised hierarchical clustering analyses using combinations of the 4 biomarkers, ACA48, U35A, U55, and U83A, to correctly identify mTBI/PTSD vs. non-TBI/PTSD cases. Accuracy represents the percentage of all mTBI/PTSD and non-mTBI/PTSD cases that were correctly diagnosed by the test, calculated as the number of correctly identified mTBI/PTSD and non-TBI/PTSD cases divided by the total number of cases analyzed. Sensitivity (true positive [TP]/[TP + false negative (FN)]) is the probability that a case predicted to have mTBI actually had it, whereas specificity (true negative [TN]/[false positive (FP) + TN]) measures the probability that a case predicted not to have mTBI did, in fact, not have it. B. Heat map of an unsupervised hierarchical clustering analysis using the combined 4 small noncoding RNA biomarkers (ACA48, U35A, U55, and U83A) showing segregation of mTBI/PTSD and non-TBI/PTSD cases. C. Receiver Operating Characteristic (ROC) analysis using the combined 4 biomarkers. ROC curve plotting the percentage of correctly identified mTBI/PTSD cases (true positive; specificity) as a function of the percentage of non-TBI/PTSD cases incorrectly identified as mTBI/PTSD cases (false positive; 1-specificity). The calculated area under the curve (AUC) is 1.0. In general, an AUC of 1 indicates the capability of a test to perfectly segregate two populations, and an AUC of 0.5 indicates that the test cannot segregate two populations beyond chance. This evidence supports the hypothesis that the combined 4 small noncoding RNA biomarker is a vigorous and sensitive test to distinguish mTBI/PTSD from non-mTBI/PTSD cases.



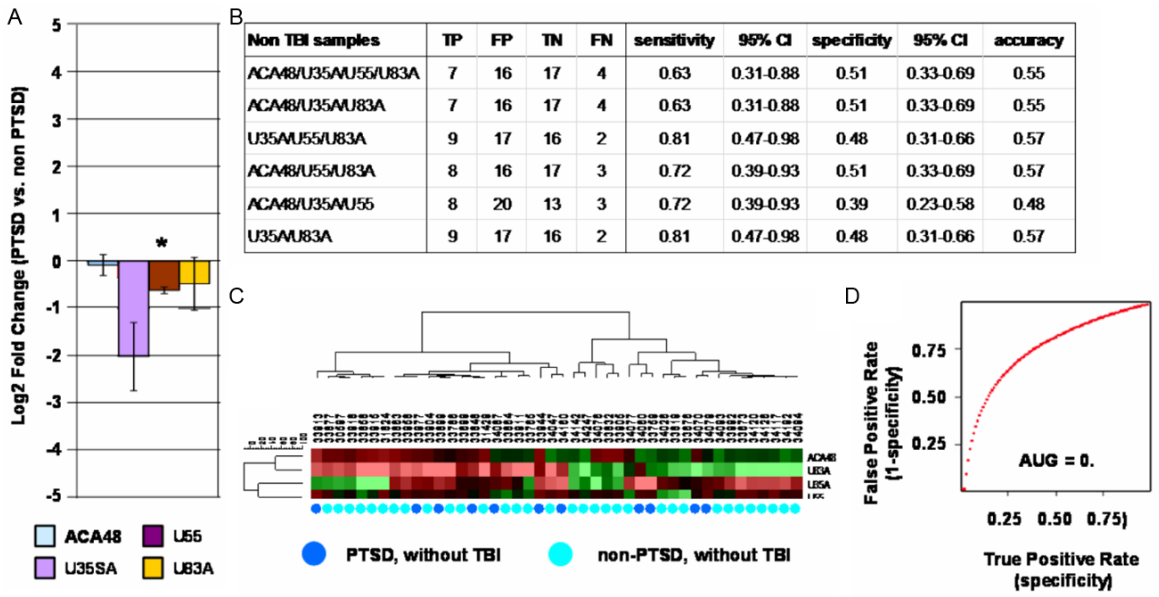
Biomarker combinations	TP	FP	TN	FN	sensitivity	95% CI	specificity	95% CI	accuracy
ACA48/U35A/U55/U83A	5	16	17	2	0.71	0.30-0.95	0.51	0.33-0.69	0.55
ACA48/U35A/U83A	5	17	16	2	0.71	0.30-0.95	0.48	0.31-0.66	0.53
U35A/U55/U83A	5	17	16	2	0.71	0.30-0.95	0.48	0.31-0.66	0.53
ACA48/U55/U83A	5	17	16	2	0.71	0.30-0.95	0.48	0.31-0.66	0.53
ACA48/U35A/U55	6	19	15	1	0.85	0.42-0.99	0.54	0.36-0.72	0.52
U35A/U83A	5	17	16	2	0.71	0.30-0.95	0.48	0.31-0.66	0.53

Figure 2. Candidate mTBI snoRNA biomarker expression in mTBI vs. non-TBI control cases in the absence of PTSD comorbidity. Contents of ACA48, U35A, U55, and U83A in PBMC from mTBI/non-PTSD and non-TBI/non-PTSD cases were assessed by qPCR. A. t-test *p*-value comparison between mTBI/non-PTSD and non-TBI/non-PTSD cases. B. Unsupervised hierarchical clustering of mTBI/non-PTSD and non-TBI/non-PTSD cases was conducted using the Unweighted Pair Group Method with Arithmetic Mean agglomerative method. Cluster assignment was based on assigning samples into either the mTBI/non-PTSD or the non-TBI/non-PTSD cluster. Presented is a summation table of unsupervised hierarchical clustering analyses using combinations of ACA48, U35A, U55, and U83A to correctly distinguish mTBI/non-PTSD vs. non-TBI/non-PTSD cases. C. Heat map of an unsupervised hierarchical clustering analysis using the combined 4 snoRNA biomarkers demonstrates a lack of effective segregation between mTBI/PTSD and non-TBI/PTSD cases. D. ROC analysis using the combined 4 biomarkers. ROC curve plotting the percentage of correctly identified mTBI/non-PTSD cases (true positive; specificity) as a function of the percentage of non-TBI/non-PTSD cases incorrectly identified as mTBI/non-PTSD cases (false positive; 1-specificity). The calculated area under the curve (AUC) is 0.64.

cases when they are not comorbid with PTSD (**Figure 2B-D**).

PBMC expression of ACA48, U35A, U55, and U83A in PTSD vs. non-PTSD cases in the absence of TBI

We also explored the potential effects of PTSD on regulation of the 4 validated snoRNA biomarkers. This study cohort contained 11 non-TBI/PTSD and 34 non-TBI/non-PTSD cases (**Table 1**). There were no significant differences between the non-TBI/PTSD and non-TBI/non-PTSD groups with respect to group average values for age (33.1 ± 11.1 and 35.4 ± 10.1 years, respectively; p=0.53), post-deployment interval (3.6 ± 2.5 and 2.6 ± 2.2 years, respectively; p=0.18), or years of education (13.3 ± 1.6 and 14.4 ± 2.2 years; p=0.14). The proportion of



Non TBI samples	TP	FP	TN	FN	sensitivity	95% CI	specificity	95% CI	accuracy
ACA48/U35A/U55/U83A	7	16	17	4	0.63	0.31-0.88	0.51	0.33-0.69	0.55
ACA48/U35A/U83A	7	16	17	4	0.63	0.31-0.88	0.51	0.33-0.69	0.55
U35A/U55/U83A	9	17	16	2	0.81	0.47-0.98	0.48	0.31-0.66	0.57
ACA48/U55/U83A	8	16	17	3	0.72	0.39-0.93	0.51	0.33-0.69	0.57
ACA48/U35A/U55	8	20	13	3	0.72	0.39-0.93	0.39	0.23-0.58	0.48
U35A/U83A	9	17	16	2	0.81	0.47-0.98	0.48	0.31-0.66	0.57

Figure 3. Candidate mTBI snoRNA biomarker expression in PTSD vs. non-PTSD control cases in the absence of TBI comorbidity. (A) PBMC contents of ACA48, U35A, U55, and U83A in PTSD/non-TBI and non-PTSD/non-TBI cases, as assessed by qPCR. Bar graphs present mean and SEM values; *p<0.05, t-test *p*-value comparison between PTSD/non-TBI vs. non-PTSD/non-TBI cases. (B, C) Unsupervised hierarchical clustering of PTSD/non-TBI and non-PTSD/non-TBI cases was conducted using the Unweighted Pair Group Method with Arithmetic Mean agglomerative method. Cluster assignment was based on assigning samples to PTSD/non-TBI and non-PTSD/non-TBI clusters. Presented is a summation table of unsupervised hierarchical clustering analyses using combinations of ACA48, U35A, U55, and U83A to correctly identify PTSD/non-TBI and non-PTSD/non-TBI cases (B). Heat map of an unsupervised hierarchical clustering analysis shows that a combination of ACA48, U35A, U55, and U83A is not effective in segregating PTSD/non-TBI vs. non-PTSD/non-TBI cases (C). (D) ROC analysis using ACA48, U35A, U55, and U83A. The calculated area under the curve is 0.75.

males in the non-TBI/PTSD and the non-TBI/non-PTSD group were 82% and 88%, respectively.

We found no significant differences in PBMC contents of ACA48, U35A, or U83A in PTSD vs. non-PTSD cases in the absence of mTBI comorbidity (**Figure 3A**). Interestingly, we did find significant down-regulation of U55 in PBMC of non-TBI/PTSD compared to non-TBI/non-PTSD. This evidence suggests that PTSD has an effect on the regulation of U55, which may contribute to the down-regulation of U55 seen in mTBI vs. non-TBI cases in the context of PTSD comorbidity (**Figure 1A**, **1B**). Nonetheless, we found that application of the 4 validated mTBI/PTSD snoRNA biomarkers, ACA48, U35A, U55, and U83A, did not have any predictive value for differentiating PTSD from non-PTSD cases in the absence of mTBI comorbidity (**Figure 3B-D**).

Regulation of the validated mTBI/PTSD snoRNA biomarkers in animal models of blast-induced mTBI or PTSD

Recently, a blast-induced rat model mTBI was developed to demonstrate the effects of air blast exposure in OEF/OIF veterans [5]. Furthermore, mice exposed to chronic social defeat are being used to model anxiety and depression symptoms seen in PTSD [17, 22]. Using the blast-induced rat mTBI model and the chronic social defeat mouse PTSD experimental model, we continued to explore potential cause-effect relationships between mTBI and/or PTSD and down-regulation of our validated snoRNA mTBI/PTSD biomarkers in PBMC. Among the 4 validated snoRNA mTBI/PTSD biomarkers of interest, rat sequence information is available only for ACA48. Therefore, we explored the regulation of ACA48 in rodent models of blast-induced mTBI or PTSD to gather proof-of-concept evidence that would show that mTBI and/or PTSD have implications on the regulation of the 4 snoRNA biomarkers.

We collected blood specimens from blast-treated rats ~1.3 years after their initial blast exposure to simulate the long-term post-deployment characteristics of the OEF/OIF cases in our initial biomarker discovery [12] and in our current biomarker validation studies. We found no change in the regulation of ACA48 in blood

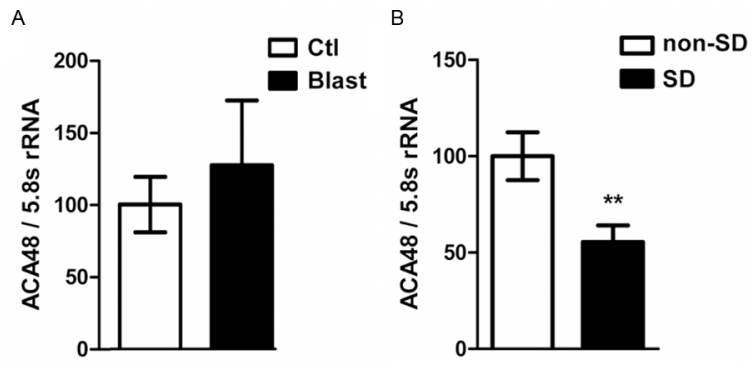


Figure 4. ACA48 snoRNA expression in blood cells from experimental rodent models of PTSD and TBI. A blast-induced mTBI rat model [5] and a chronic social defeat (SD) mouse model of PTSD [17] were used in these studies. Contents of ACA48 snoRNA biomarker in blood cells were analyzed by qPCR using primer sets designed for targeting rat or mouse ACA48. A. Expression of ACA48 in blood cells from rats exposed to blast-mediated TBI rats compared to control rats. Blood specimens were collected ~1.3 years after initial blast exposure. B. Expression of ACA48 in blood cells from mice subjected to SD compared to control mice. Blood specimens were collected within a short time (~24 h) after completion of the chronic SD protocol. A, B. Bar graphs represent ACA48 content normalized to those for 5.8S rRNA using the $2^{-\Delta\Delta Ct}$ method. Levels of target small non-coding RNA were expressed relative to those in the control groups. T-test, *p<0.05.

specimens from the blast-induced mTBI rat model compared to control rats (**Figure 4A**), implicating that the down-regulation of ACA48 that we observed in veterans with comorbid mTBI and PTSD (**Figure 1A**, **1B**) might not be due to exposure to blast-induced mTBI. Further experimental assessments evaluating the potential impact of blast exposure on the regulation of U35A, U55, and U83A in the blast-induced mTBI rat model will have to wait for the availability of rat sequence information for these snoRNA biomarkers.

Exploring the potential influence of PTSD on the regulation of ACA48, we observed a significant down-regulation of ACA48 (by approximately 50%) in blood cells of mice exposed to chronic social defeat (SD) compared to control mice (**Figure 4B**). Our observation demonstrates, for the first time, that experimental PTSD is associated with altered regulation of a specific snoRNA, implicating that the presence of PTSD may contribute to the observed down-regulation of ACA48 in veterans with comorbid mTBI and PTSD. We note that our experimental evidence of down-regulation of ACA48 was based on monitoring the PTSD mouse model within a short time frame (by 24 hours) after completion of the chronic social defeat protocol. Interestingly, we found no observable changes in the content of ACA48 in PTSD vs. non-PTSD cases in the absence of TBI long after the veterans' deployment (average post deployment interval of 3.6 years) (**Figure 3A**), suggesting that while PTSD may have a direct acute impact on the regulation of ACA48 in PBMC, this effect may not last over the long-term. Additional studies will be required to test longer-term impacts of PTSD on the regulation of ACA48, U35A, U55, and U83A in this PTSD mouse model.

Discussion

Validation of select snoRNA biomarkers for distinguishing veteran mTBI cases with or without PTSD comorbidity

We confirmed that 4 snoRNA species, ACA48, U35A, U55, and U83A, are significantly down-regulated in PBMC specimens from mTBI/PTSD compared to control, non-TBI/PTSD cases in two independent study cohorts. Moreover, we established the ability of these 4 validated biomarkers to distinguish cases with or without mTBI in the context with PTSD comorbidity. Identification of these snoRNA biomarkers will provide for improved detection of co-morbid mTBI and PTSD and for more sensitive measurements for clinical trials.

We observed that the 4 validated snoRNA mTBI/PTSD biomarkers were not differentially regulated among mTBI and non-TBI in the absence of PTSD comorbidity, suggesting that the presence of mTBI alone is insufficient to induce the down-regulation of ACA48, U35A, U55, and U83A seen in cases of comorbid mTBI and PTSD. Consistent with this, our ongoing studies observed no detectable change in contents of ACA48 in blood cells from a blast-induced TBI rat model.

We found significantly lower contents of U55 in PBMC from non-TBI/PTSD vs. non-TBI/non-PTSD, but no detectable changes in the regulation of ACA48, U35A, or U83A in PBMC

of OEF/OIF veterans with PTSD compared to veterans without PTSD in the absence of TBI comorbidity long after their deployment. Interestingly, our ongoing studies using the chronic social defeat PTSD mouse model suggest that PTSD may lead to down-regulation of ACA48 over the short-term. This may reflect a short-term, acute response to trauma exposure, since we found no changes in the regulation of ACA48 in veteran PTSD subjects 3.6 years post-deployment. Additional investigations will be necessary to explore the short- and long-term contributions of PTSD on the down regulation of ACA48, U35A, U55, and U83A in veterans with comorbid mTBI and PTSD.

Our evidence suggests that the presence of mTBI alone is insufficient to induce down-regulation of these snoRNA biomarkers, but the presence of PTSD may contribute to the down-regulation of U55 and ACA48 seen in mTBI veterans with comorbid mTBI and PTSD. Our observations suggest that biological interactions between TBI and PTSD may contribute to the clinical features of mTBI with comorbid PTSD.

Most mTBI cases among veterans of the conflicts in Afghanistan and Iraq are due to blast-induced injuries. However, we do not have any information on our study cohort in regards to the type of injury—blast versus non-blast—that individual cases were exposed to. Future studies will be necessary to explore the impact of blast and non-blast TBI injuries on the regulation of these snoRNA biomarkers.

Potential relevance of the four snoRNA biomarkers to TBI/PTSD pathophysiology

The 4 small noncoding RNA mTBI/PTSD biomarkers that we validated in our present studies are all members of the small nucleolar RNA (snoRNA) class. SnoRNAs are known for their methylation and pseudouridylation of other small noncoding RNAs, particularly ribosomal RNAs, transfer RNAs, and small nuclear RNAs [23]. Methylation/pseudouridylation of small noncoding RNAs may modulate the folding of small noncoding RNAs and protect small noncoding RNAs from hydrolysis [19]. Recent evidence demonstrates that select snoRNA (snoRNA HBII-52) is also involved in alternative splicing of the serotonin receptor 2C [20]. Changes in the regulation of snoRNAs have been associated with multiple cancers, and deletion of imprinted snoRNAs in chromosome 15q11-q13 has been associated with Prader-Willi syndrome [21]. There is, however, little information on the potential biological activities of ACA48, U35A, U55, and U83A.

Recent evidence revealed that certain snoRNAs, referred to as sno-miRNAs, may also exhibit microRNA (miRNA) functions by interfering with translation and/or by promoting degradation of targeted mRNAs [24]. Interestingly, one of our snoRNA biomarkers, U83A, is among the sno-miRNAs reported to exert sno-miRNA activities [24]. Ongoing studies are attempting to characterize specific gene targets of U83A.

Another snoRNA biomarker validated in our present study, U35A, has been shown to be effective in modulating cellular responses to oxidative stress and inflammatory mediators. It has also been demonstrated that knockdown of U35A mitigates lipopolysaccharides-induced oxidative stress in the liver [25]. While the mechanisms of action are presently unknown, these observations suggest that down-regulation of U35A in PBMC of veterans with comorbid TBI and PTSD may reduce resilience to inflammatory stress, leading to the promotion of oxidative stress conditions and contributing to mTBI and/or PTSD clinical features over the long-term.

Conclusions

Collectively, evidence from our studies suggest that additional application of the 4 snoRNA biomarker to current diagnostic criteria may provide an objective biomarker pattern to help identify veterans with comorbid mTBI and PTSD. Furthermore, future studies clarifying the potential bioactivity of ACA48, U55, and U83A will provide additional insight on pathogenic mechanisms contributing to long-term clinical complications in veterans with comorbid TBI/PTSD. Moreover, observations from our studies suggest that future investigations on mTBI biomarkers, as well as pathogenic mechanisms underlying mTBI, should carefully consider the impact of interactions with PTSD.

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Disclosure of conflict of interest

None to declare.

Abbreviations

Afr Am, African American; AUG, area under the curve; Bl, black; cDNA, complementary DNA; DVANJHCS, Department of Veterans Affairs, Bew Jersey Health Care System; FP, false positive; Hisp, Hispanic; mTBI, mild traumatic brain injury; nHisp, non-hispanic; OEF, Operation Enduring Freedom; OIF, Operation Iraqi Freedom; PTSD, post-traumatic stress disorder; PBMC, peripheral blood mononuclear cells; qPCR, real-time quantitative polymerase chain reaction; RBANS, Repeatable Battery for Neuropsychological Testing; ROC, Receiver Operating Characteristic; snoRNP, small nucleuolar ribonucleoprotein; snoRNA, small nucleolar RNA; TBI, traumatic brain injury; TP, true positive; VA-BIST, Veteran Traumatic brain injury screening tool; Wh, white; WRIIC, War Related Illness and Injury Study Center.

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